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| <b>13. SUPPLEMENTARY NOTES</b>  |                         |                                 |   |  |   |
| <b>14. ABSTRACT</b><br>We have found that the FGFR-4 Arg388 allele is strongly associated with the occurrence of prostate cancer and with metastasis and PSA recurrence in men undergoing radical prostatectomy. We will test the role of FGFR-4 in prostate cancer metastasis using autochthonous and orthotopic mouse models of cancer. We will also identify changes in gene expression due to the presence of the FGFR-4 Arg388 allele that lead to the metastatic phenotype. To this end we have established and carried out the characterization of transgenic mice with prostatic specific expression of FGFR-4 Arg388 and Gly388; constructed a FGFR-4 siRNA lentivirus and a PC3 and LNCaP siRNA cell lines and identified Ehm2 as an FGFR-4 Arg388 regulated gene and characterized its role in prostate cancer. These key reagents will allow us to evaluate the effects of FGFR-4 on prostate cancer progression in vivo. Further studies of FGFR-4 Arg388 regulated gene expression in prostate cancer tissues are underway. |                         |                                 |   |  |   |
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## INTRODUCTION

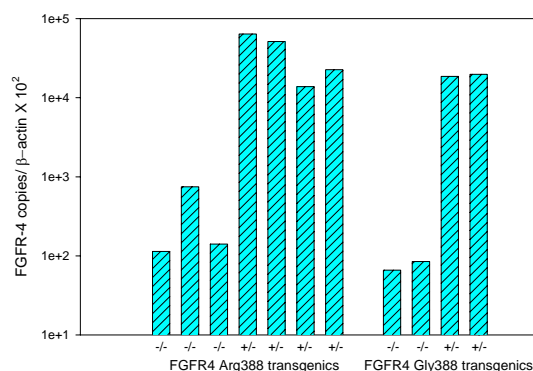
Fibroblast growth factors (FGFs) can control a multitude of cellular processes including proliferation, differentiation, survival, motility and angiogenesis. FGF2, FGF6, FGF8 and FGF17 are all expressed at increased levels in human prostate cancer and bind to FGF receptor-4 (FGFR-4) with high affinity. However, the role of FGFR-4 in human prostate cancer has not been systematically examined to date. Studies have shown that a germline polymorphism of the FGFR-4 gene resulting in expression of arginine at codon 388 (Arg<sup>388</sup>) is associated with aggressive disease in patients with breast and colon cancers and sarcomas. We have found that homozygosity for the FGFR-4 Arg<sup>388</sup> allele is strongly associated with the occurrence of prostate cancer in white men and the presence of the FGFR-4 Arg<sup>388</sup> allele is also correlated with the occurrence of pelvic lymph node metastasis and PSA recurrence in men undergoing radical prostatectomy (1). Pooled cell lines expressing predominantly the FGFR-4 Arg<sup>388</sup> or Gly<sup>388</sup> allele were established by stable transfection in immortalized prostatic epithelial cells. Expression of FGFR-4 Arg<sup>388</sup> resulted in increased cell motility and invasion through Matrigel when compared to cells expressing the FGFR-4 Gly<sup>388</sup> allele. Microarray analysis has revealed that urokinase plasminogen activator system, matrix metalloproteases and cathepsin proteases may be involved the increased motility and increased metastasis seen in cells bearing the Arg<sup>388</sup> allele. We hypothesize that FGFR-4, and in particular the FGFR-4 Arg<sup>388</sup> variant, plays an important role in prostate cancer progression and metastasis based on correlative studies in humans. We propose to test this hypothesis in autochthonous and orthotopic mouse models of cancer. Furthermore, we hypothesize that these effects occur through specific changes in gene expression so we will carry out studies to identify changes in gene expression due to the presence of the FGFR-4 Arg<sup>388</sup> allele that lead to the metastatic phenotype.

## BODY

As outlined in our Statement of Work, we will seek to accomplish nine tasks in our three years of funding. We have accomplished or made substantial progress on all of these tasks.

### Task 1: Establish and characterize pathology in transgenic mice with strong prostate specific expression of FGFR-4 Arg<sup>388</sup> and Gly<sup>388</sup> variants (Months 1-18)

We have established FGFR-4 transgenic mice by injection of FGFR-4 Arg<sup>388</sup> and Gly<sup>388</sup> cDNAs under the control of the strong prostate specific ARR2Pb promoter into mouse blastocysts. We have performed quantitative RT-PCR on RNAs extracted from the prostates of these mice. As can be seen in Figure 1, expression of FGFR-4 is approximately 100-fold higher in the transgenic mice (note that graph is log scale).



**Figure 1. Expression of FGFR-4 in transgenic mice.**

Expression of FGFR-4 was determined by quantitative RT-PCR in prostates from transgenic mice expressing FGFR-4 under the control of the prostate specific ARR2-Pb promoter. Note log scale.

After establishment of transgenic lines, we sacrificed mice at 2 month intervals and performed full necropsies to evaluate for any pathology, in particular, prostatic intraepithelial neoplasia or adenocarcinoma of the prostate. Analysis reveals hyperplastic and dysplastic lesions in the ventral and dorsolateral prostate of transgenic mice but no invasive carcinoma has been identified to date. As expected, no pathology has been seen in other organs, since the ARR2Pb promoter is prostate specific.

**Task 2: Cross FGFR-4 transgenics with TRAMP and ARR2Pb-myc mice and evaluate tumor progression (Months 12-36)**

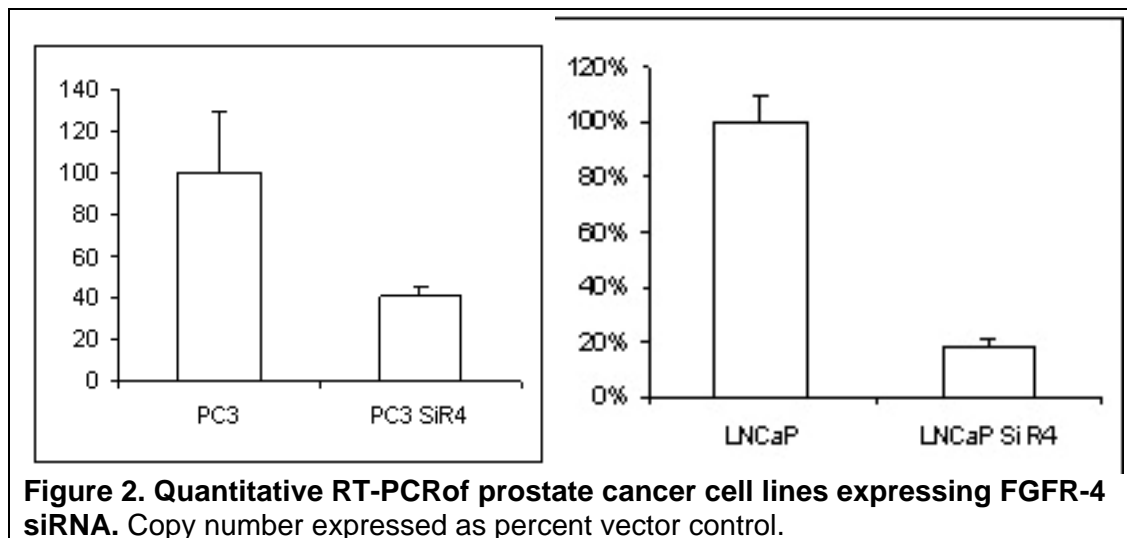
We have initiated the crosses of our FGFR-4 transgenic mice and the TRAMP or ARR2Pb-myc mice. We currently have a large number of mice resulting from both of these crosses and will carry out an interim analysis shortly.

**Task 3: Collect tumors and establish cell lines from WT TRAMP and bitransgenic mice and evaluate effect of FGFR-4 Arg<sup>388</sup> and Gly<sup>388</sup> on proliferation, apoptosis and motility (Months 12-36)**

Task 3 is underway (see Task 2, above).

**Task 4: Establishment and characterization of LNCaP and PC-3 cell lines with markedly decreased FGFR-4 expression by RNAi (Months 1-12)**

We have constructed a lentivirus vector containing a SiRNA encoding sequence for FGFR-4. PC3 and LNCaP prostate cancer cells have been infected and selection performed. By quantitative RT-PCR these cell have 60% and 80% decreases in FGFR-4 mRNA, respectively (Fig 2). By Western blot there is a marked decrease in FGFR-4 protein in the siRNA expressing PC3 cells (Fig 3). No significant affect on cell proliferation of FGFR-4 siRNA expression was seen (data not shown), consistent with our earlier observations in PNT1a cells overexpressing FGFR-4 variants.



**Figure 3. Western blot with anti-FGFR-4 antibody.**

PC3: controls infected with empty virus; PC3 siRNA: PC3 infected with siRNA containing virus. 293 cells were transiently transfected with FGFR-4 Arg<sup>388</sup> or Gly<sup>388</sup> or empty vector and used for Western blot to demonstrate antibody specificity.

Task 5: Determination of the effect of decreased FGFR-4 expression in an orthotopic injection model of prostate cancer metastasis (Months 12-24)

We have begun to assess the *in vivo* behavior of the cell lines established in Task 4, above, using an orthotopic injection model in nude mice in which cells are injected directly into the prostate. We have carried out orthotopic injections and have performed necropsies. Tissues have been submitted for sectioning and we will perform pathological analysis shortly.

Task 6. Analysis of tumors from orthotopic injection model (Months 24-36)

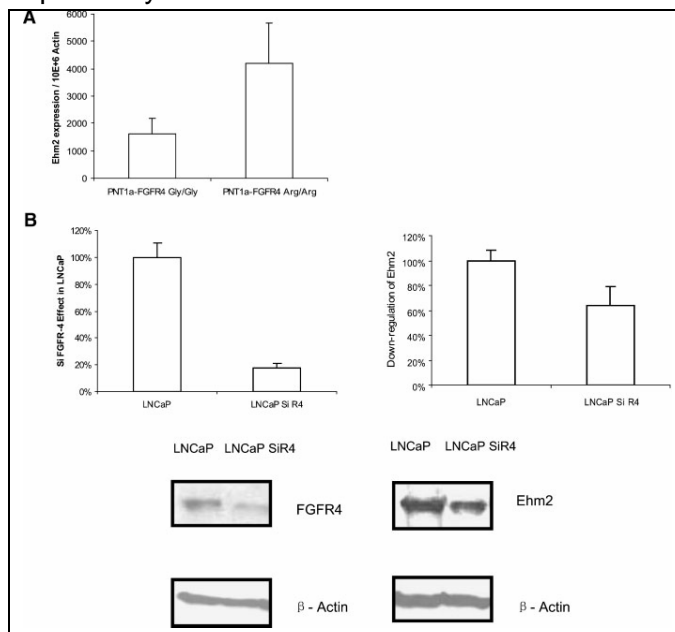
This task is dependent on Task 5, above, and will begin shortly after sections are obtained.

Task 7: Evaluation of expression of candidate proteins mediating the biological effects of FGFR-4 Arg<sup>388</sup> and correlation with FGFR-4 genotype in white patients with prostate cancer (Months 1-18)

We have been assembling our tissue samples and have made protein extracts and will begin ELISAs of PAI-1, TIMP-1 and cystatin C shortly. It is far more cost effective to batch this analysis since the ELISAs need multiple wells for standards and controls. This approach also minimizes experimental variability.

Task 8: Identification of additional candidate genes mediating the biological effects of FGFR-4 Arg<sup>388</sup> using targeted microarrays (Months 18-24)

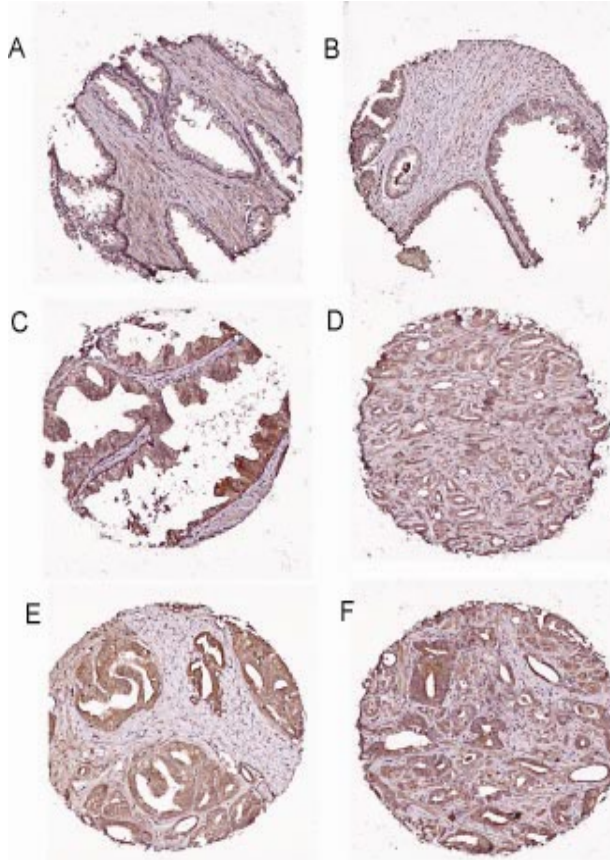
We have identified a potential candidate gene that may mediate some of the biological effects of the FGFR-4 Arg<sup>388</sup> polymorphic variant by analysis of expression microarrays. The Ehm2 gene is a member of the NF2/ERM/4.1 superfamily. Members of this superfamily all contain a FERM domain and are involved in membrane-cytoskeletal



interactions. There is a strong correlation between Ehm2 gene expression and the metastatic potential of K-1735 and B16 melanoma cells (2). It is proposed that alterations of the expression level of Ehm2 are likely to be linked to one or more steps of cancer metastasis through regulation of interaction between cell surface transmembrane proteins and cytoskeletal proteins. Ehm2 is expressed at higher levels in PNT1A cells expressing FGFR-4 Arg<sup>388</sup> when compared to cells expressing equivalent amounts of FGFR-4 Gly<sup>388</sup> (Figure 4A) and knockdown of FGFR-4 decreases Ehm2 (Fig 4B).

**Figure 4. FGFR-4 increases Ehm2 expression. A.** Expression was quantitated using real-time RT-PCR of RNAs from PNT1a cells overexpressing the Arg<sup>388</sup> or Gly<sup>388</sup> variant. Data was normalized using  $\beta$ -actin transcript levels. **B.** SiRNA against FGFR-4 decreases FGFR4 and Ehm2 expression in LNCaP cells at the RNA and protein level

To analyze expression of Ehm2 at the protein level *in vivo* we performed immunohistochemical analysis of Ehm2 expression using a prostate cancer tissue microarray containing 32 matched normal and prostate cancer tissues. Staining was predominantly seen in benign and malignant epithelial cells (Figure 5) with some staining of smooth muscle. Stained slides were digitized and scored both for extent of staining



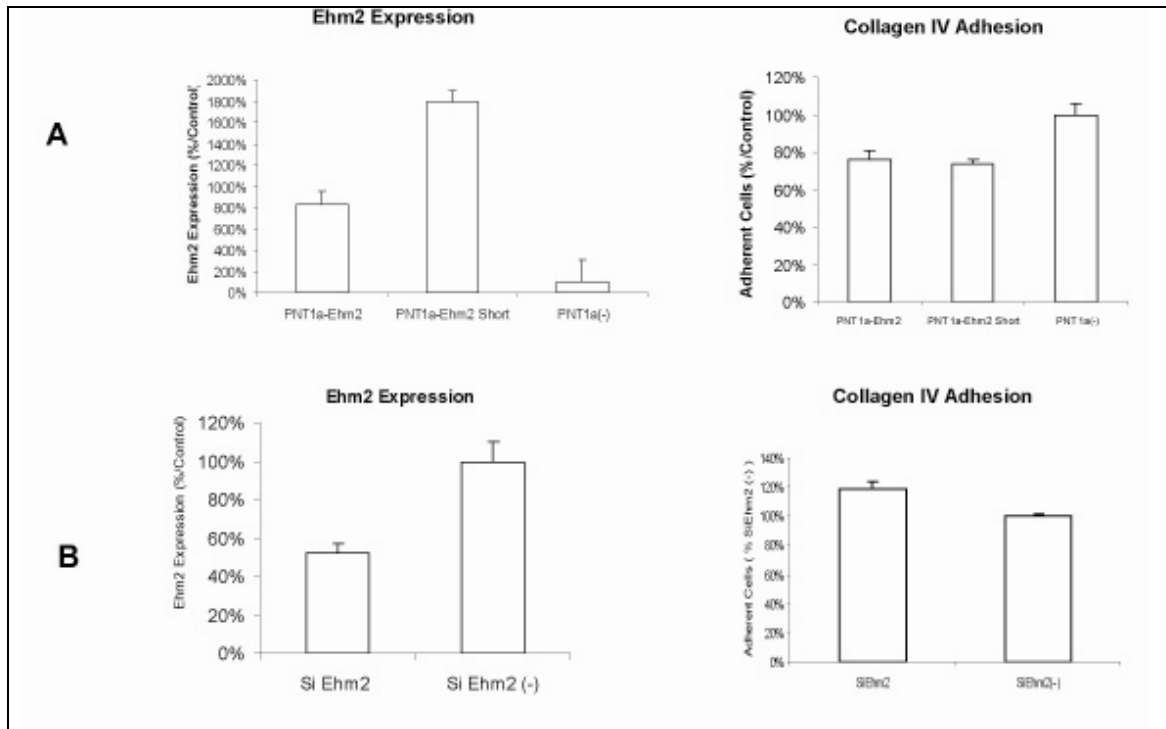
(scale of 0-3) and intensity of staining (scale of 0-3). An average staining index was calculated from the extent of staining score for the three cores multiplied by the staining intensity score so that the staining index ranged from 0 (no staining) to 9 (extensive, strong staining). Normal prostate epithelium showed a staining index of  $6.3 \pm 0.38$  (mean  $\pm$  SEM) while the cancer tissues had a staining index  $7.4 \pm 0.29$ . This difference was statistically significant ( $p < 0.05$ , Mann Whitney). Overall, 27% of normal tissues showed low staining (index  $< 6.0$ ) while only 10% of cancers had low staining. In contrast, 40% of cancers showed maximal staining intensity (index = 9) Vs 8% of normal epithelium. Of note was the finding that none of the cases with low Ehm2 staining (staining index  $< 6.0$ ) had a PSA recurrence, while all cases with recurrence had moderate to strong Ehm2 staining (staining index 6-9). This difference is statistically significant ( $p = 0.03$ , Fisher exact test).

**Figure 5. Expression of Ehm2 in normal prostate and prostate cancer.**

Immunohistochemistry with anti-Ehm2 antibody was performed using a tissue microarray of normal and prostate cancer tissues. A. Normal prostate with weak staining of epithelium. B. Normal prostate with moderate epithelial staining. C. High grade prostatic intraepithelial neoplasia with strong staining. D. Prostate cancer with moderate staining. E, F. Prostate cancers with strong staining.

Ehm2 may regulate cytoskeleton interactions with transmembrane proteins and thus affect cancer cell adhesion. To evaluate the impact of Ehm2 on adhesion, we carried out two series of complementary experiments. PNT1a cells, which express low levels of Ehm2, were transiently transfected with an expression vectors containing the Ehm2 cDNA. Two cDNAs were used: a full-length form and a 5' truncated variant that would give rise to a smaller protein that is the main protein seen in prostate epithelial cells ((3); see attached manuscript for details). The results of these experiments are shown in Figure 6A. Ehm2 expression was increased 8 or 17-fold in the transfected cells and this was associated with a 30% decrease in adhesion to collagen IV. We then decreased Ehm2 mRNA in the LAPC4 cell line, which showed the second strongest Ehm2 expression among tested prostate cell lines, using siRNA. As shown in Fig 6B, 24h after

siRNA transfection, there was an approximately 50% decrease in Ehm2 transcript level, while adhesion to collagen IV increased about 20% compared to control cells. These results indicate that Ehm2 expression is associated with decreased adhesion to collagen IV by prostatic epithelial cells. Collagen IV adhesion has been previously shown to be inversely correlated with metastatic behavior in breast cancer (4). All experiments were repeated 3 times and ranges are shown.



**Figure 6. Ehm2 expression results in decreased collagen IV adhesion**

**A.** PNT1A cells were transiently transfected with full length or 5' truncated Ehm2 cDNA cloned into the pCMV-Tag2b expression vector or empty vector. Expression of Ehm2 in transfectants by quantitative RT-PCR (left). Collagen IV adhesion of Ehm2 and control PNT1A transfected cells (right). One of 3 experiments with similar results is shown.

**B.** LAPC4 prostate cancer cells were transiently transfected with Ehm2 or control siRNA. Expression of Ehm2 in transfectants by quantitative RT-PCR (left) Collagen IV adhesion of Ehm2 siRNA and control siRNA transfected LAPC4 cells (right). Range of triplicate determinations is shown.

In summary, we have identified Ehm2 as a gene which can promote metastasis that is upregulated by expression of the FGFR-4 Arg<sup>388</sup> allele. A manuscript describing these findings (including additional data not presented in this report) was recently published in *Prostate* (3) and a copy is appended.

We are also actively trying to identify and characterize additional FGFR-4 Arg<sup>388</sup> regulated genes. To this end, we have compared gene expression in the PNT1a Arg<sup>388</sup> and Gly<sup>388</sup> overexpressing cell lines using Agilent Human Whole Genome Oligonucleotide Arrays. These arrays contain oligonucleotides (approximately 41,000) corresponding to the known human transcriptome. A relatively limited set of genes were identified using this analysis. A total of 30 genes were upregulated 2.2-fold or greater in cell overexpressing the FGFR-4 Arg<sup>388</sup> and 28 were downregulated by a similar amount.



We are currently evaluating the most promising candidate gene: phosphatidylyl-4-phosphate 5-kinase (PIP5K). PIP5K is increased 2.4-fold in FGFR-4 Arg<sup>388</sup> expressing prostate cells. This enzyme can catalyze formation of phosphatidyl-inositol 4,5 biphosphate (PIP<sub>2</sub>) and thus can increase the concentration of this key lipid in cells. PIP<sub>2</sub> can modulate actin polymerization and have a variety of other effects including protection against apoptosis and alterations in membrane vesicle trafficking (5). PIP<sub>2</sub> availability may also alter PI-3 kinase activity and hence AKT activation. Thus this enzyme may potentially modulate several process associated with more aggressive tumor behavior. We are seeking to confirm this observation and understand its implications for the role of FGFR-4 in prostate cancer progression.

Task 9: Evaluation of expression of candidate proteins mediating the biological effects of FGFR-4 Arg<sup>388</sup> and correlation with FGFR-4 genotype in white patients with prostate cancer (Months 24-36)

See Task 8 above. These experiments are planned for year three and have been partially completed for Ehm2.

### **KEY RESEARCH ACCOMPLISHMENTS**

- \* Establishment and initial characterization of FGFR-4 Arg<sup>388</sup> and Gly<sup>388</sup> transgenic mice.
- \* Mating of FGFR-4 transgenic mice to TRAMP and ARR2-Pb myc transgenic mice.
- \* Construction of FGFR-4 siRNA lentivirus and establishment of PC3 and LNCaP FGFR-4 siRNA cell lines
- \* Identification of Ehm2 as an FGFR-4 Arg<sup>388</sup> regulated gene and characterization of its role in prostate cancer.
- \* Identification of PIP5K as potential modulator of FGFR-4 Arg<sup>388</sup> activity in prostate cancer.

### **REPORTABLE OUTCOMES**

- \* Establishment of FGFR-4 Arg<sup>388</sup> and Gly<sup>388</sup> transgenic mouse lines
- \* Establishment of PC3 and LNCaP FGFR-4 SiRNA cell lines
- \* Publication of manuscript describing role of Ehm2 in prostate cancer ((3) see attached)

### **CONCLUSION**

Our published data indicates that FGFR-4 plays an important role in prostate cancer initiation and progression. We have developed key reagents that will allow us to assess the biological effects of FGFR-4 on prostate cancer progression *in vivo*. We have also identified Ehm2 as a metastasis promoting gene that is upregulated by expression of the FGFR-4 Arg<sup>388</sup> variant. Further studies of this gene, as well as PAI-1, TIMP-1, cystatin C and PIP5K in men with prostate cancer will yield new insights into the mechanism by which the FGFR-4 Arg<sup>388</sup> can promote prostate cancer progression.

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# Increased Expression of the Metastasis-Associated Gene Ehm2 in Prostate Cancer

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**BACKGROUND.** Alterations of fibroblast growth factors and their receptors contribute to prostate cancer progression by enhancing cell survival, motility, and proliferation. The expression of the FGFR-4 Arg<sup>388</sup> variant is correlated with the occurrence of pelvic lymph node metastasis and biochemical (PSA) recurrence in men undergoing radical prostatectomy. Ehm2 is an androgen-regulated gene that has been associated with metastasis in other systems, so we sought to determine if it is expressed in prostate cancer and if the FGFR-4 Arg<sup>388</sup> variant can increase its expression.

**METHODS.** Expression of Ehm2 was examined by quantitative RT-PCR and Western blotting in prostate cell lines and by quantitative RT-PCR, in situ hybridization, and immunohistochemistry in prostate tissues. The effect of Ehm2 expression on collagen IV adhesion was tested by transient overexpression and RNA interference.

**RESULTS.** Ehm2 expression is upregulated in prostate cancer cell lines and prostate cancer tissues. Expression of the FGFR-4 Arg<sup>388</sup> variant results in increased expression of Ehm2. Increased expression of Ehm2 leads to decreased adhesion to collagen IV, which has been associated with metastasis in cancers. Analysis of tissue microarrays revealed that increased Ehm2 expression is associated with biochemical recurrence after radical prostatectomy, which is indicative of more aggressive disease.

**CONCLUSIONS.** Ehm2 is overexpressed in prostate cancer and may enhance disease progression and metastasis. *Prostate* 66: 1641–1652, 2006. © 2006 Wiley-Liss, Inc.

**KEY WORDS:** Ehm2; prostate cancer; recurrence; cell adhesion; metastasis

## INTRODUCTION

Prostate cancer is the most common visceral malignancy in US men, with approximately 230,000 new cases and 29,000 deaths in 2004 [1]. Prostate cancer deaths are a result of metastatic disease and treatment of such metastatic disease is one of the major therapeutic challenges in prostate cancer treatment. Many studies have been focused on identification of the biological mechanisms of metastasis in prostate cancer and significant insights into the basis of prostate cancer metastasis have emerged from these studies [2–7]. Malignant tumors are heterogeneous with regard to metastatic potential, and prostate cancer cells with high- and low-metastatic potential vary in their biological properties, such as proliferation,

adhesiveness, invasiveness, and motility. This variation is a result of both germline variation between

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individuals [7] and somatic alterations of genes and gene expression in cancer cells [2–6]. Despite recent advances, our understanding of the process by which prostate cancer cells metastasize to distant sites remains imperfect.

Fibroblast growth factors (FGFs) can control a multitude of cellular processes including proliferation, differentiation, survival, motility, and angiogenesis. FGF2, FGF6, FGF8, and FGF17 are all expressed at increased levels in human prostate cancer and bind to FGF receptor-4 (FGFR-4) with high affinity [8]. Studies have shown that a germline polymorphism of the *FGFR-4* gene resulting in expression of arginine at codon 388 (Arg<sup>388</sup>) is associated with aggressive disease in patients with breast and colon cancer [9] as well as other malignancies [7,10,11], although in some patient populations, these associations have not been present for breast and colon cancer [12,13]. We have found that the presence of the FGFR-4 Arg<sup>388</sup> allele is correlated with the occurrence of pelvic lymph node metastasis and biochemical (PSA) recurrence in men undergoing radical prostatectomy [7]. Pooled cell lines expressing predominantly the FGFR-4 Arg<sup>388</sup> or Gly<sup>388</sup> allele were established by stable transfection in immortalized prostatic epithelial cells (PNT1a cells), and expression of FGFR-4 Arg<sup>388</sup> variant resulted in increased cell motility and invasion through Matrigel when compared to cells expressing the FGFR-4 Gly<sup>388</sup> variant. Further analysis of PNT1a cells overexpressing the FGFR-4 Arg<sup>388</sup> variant revealed that increased expression of the urokinase plasminogen activator receptor may be involved in the increased metastasis seen in cells bearing the Arg<sup>388</sup> allele. It is likely that other genes may also be involved in increasing the metastatic potential of FGFR-4 Arg<sup>388</sup> expressing prostate cancer cells.

One gene that has been linked to metastasis is *Ehm2*. The *Ehm2* gene is a member of the NF2/ERM/4.1 superfamily [14]. Members of this superfamily all contain a FERM domain and are involved in membrane–cytoskeletal interactions. Hashimoto et al. [15] demonstrated a good correlation between *Ehm2* gene expression and the metastatic potential of mouse K-1735 and B16 melanoma cells. They proposed that alterations of the expression level of *Ehm2* are likely to be linked to one or more steps of cancer metastasis through regulation of interaction between cell surface transmembrane proteins and cytoskeletal proteins [15]. Human *Ehm2* was recently characterized in a human fibrosarcoma cell line model of steroid-regulated cytoskeletal reorganization and shown to be androgen-regulated [16]. Moreover, a related FERM domain protein, ezrin, was reported to be androgen-regulated in the rat prostate [17] and also overexpressed in metastatic rhabdomyosarcomas [18]. Since FGFR-4 is a

transmembrane protein that can interact with multiple other cell surface proteins, we sought to determine if expression of the FERM domain protein *Ehm2* is increased in prostate cancer cells containing the FGFR-4 Arg<sup>388</sup> variant.

We report here that expression of the FGFR-4 Arg<sup>388</sup> variant results in increased expression of *Ehm2* in prostate epithelial cells. To further characterize the function of *Ehm2* in prostate cancer, we carried out a systematic study of *Ehm2* expression by quantitative real-time PCR and immunohistochemistry. *Ehm2* expression is upregulated in prostate cancer cell lines and prostate cancer tissues. Increased expression of *Ehm2* leads to decreased adhesion to collagen IV, which has been associated with metastasis in cancers. Analysis of tissue microarrays revealed increased *Ehm2* expression that is associated with biochemical recurrence following radical prostatectomy, which is indicative of more aggressive disease. Thus, *Ehm2* is overexpressed in prostate cancer and may enhance disease progression and metastasis.

## MATERIALS AND METHODS

### Tissue Culture

PNT1a, an immortalized normal prostatic epithelial cell line, was obtained from the European Collection of Animal Cell Cultures (Porton Down, United Kingdom). PC3 and LNCaP cell lines were obtained from the American Type Culture Collection. All cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. The LAPC4 cell line, which was established from the LAPC4 human prostate cancer xenograft [19], was kindly provided by Dr. Charles Sawyers (University of California, LA). It was maintained in 1× Iscoves (Invitrogen) with 10% FBS, 1% PenStrep (Invitrogen), 1% L-Glutamine (Invitrogen), and supplemented with 10 nM R1881 (Perkin Elmer Life Sciences, Boston, MA).

### Human Prostate Tissue Samples

All samples of human prostate tissues were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate cancer tissue bank. Fresh frozen tissue punches of normal and tumor tissue were obtained at the time of radical prostatectomy as described previously [20]. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 70–100%. Normal peripheral zone and hyperplastic transition zone samples were free of tumor.

### RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

RNA was extracted as described previously [21] from 86 tissue samples collected from radical prostatectomies by Baylor Prostate SPORC Tissue Core. There were 19 normal samples (peripheral zone tissue), 20 early recurrence cancers (PSA recurrence in less than 1 year), 19 late recurrence cancers (PSA recurrence after 1–5 years), 20 non-recurrent samples (no PSA recurrence in 5 years), and 8 benign prostate hyperplasia (BPH) samples. Primers for Ehm2 were located between exon 8 and 11 (Genebank number: AB032179) and were as follows: Ehm2 Forward: 5'-GCCAAGTGGCTGGAAAT GTATG-3' Reverse: 5'-GAAGAATGCGTGG TGCTCAAC-3'. Control  $\beta$ -Actin primer has been described previously [21]. Five micrograms of each RNA sample was reversed transcribed into cDNA in a total volume of 100  $\mu$ l by using iScript cDNA Synthesis kit (BioRad Laboratories, Hercules, CA) according to the manufacturer's protocol. Real-time PCR was carried out in a final reaction volume of 25  $\mu$ l by using 5  $\mu$ l of the template cDNA or standard control vector which contained genes of interest. The Mastermix for real-time PCR contained 2 mM  $MgCl_2$ , 0.4  $\mu$ M each forward and reverse primers, and 2.5  $\mu$ l of LC-FastStart DNA Master SYBR GREEN (10 $\times$ ; Roche, Indianapolis, IN). Real-time PCR was done by using the iCycler IQ instrument from BioRad.  $\beta$ -Actin, Genebank Access BC004251, plasmid (ATCC#MGC-10559) was purchased from American Type Culture Collection. The Ehm2 plasmid was constructed into PCR 2.1-Topo vector from Topo TA Cloning Kit (Invitrogen). Plasmids were prepared by using the Qiagen Spin Mini-prep Kit (Qiagen, Inc., Valencia, CA). Quantification of plasmid was performed using a UV/Visible spectrophotometer. The plasmid concentration was converted into copy number, a dilution series of each plasmid from 10<sup>8</sup> to 10<sup>2</sup> were used as DNA standard for real-time PCR. All real-time PCR efficiencies were controlled in the range of 100%  $\pm$  5.

### Western Blotting

Total protein was extracted from cells using RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). For Western blots, 40  $\mu$ g of protein extract/lane were electrophoresed, transferred to nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Piscataway, NJ), and incubated overnight with a 1:200 dilution of anti-Ehm2 F19 goat polyclonal antibody (Santa Cruz), which was raised against a peptide mapping an internal region of EHM2 of human origin, a 1:1,000 dilution of anti-Flag M2 monoclonal antibody (Stratagene), an anti-FGFR4 antibody (1:500; Santa

Cruz) or anti- $\beta$ -actin (1:5,000, Sigma). Membranes were washed and treated with mouse anti-goat IgG (1:5,000; Santa Cruz), mouse anti-rabbit (1:10,000, Santa Cruz) or goat anti-mouse IgG secondary antibody (1:5,000, Pierce Biotechnology, Rockford, IL) conjugated to horseradish peroxidase. The antigen-antibody reaction was visualized using an enhanced chemiluminescence (ECL) assay (Amersham Biosciences) and exposed to ECL film (Amersham Biosciences).

### In Situ Hybridization of Prostate Tissues

The human Ehm2 sequence was obtained by PCR using primers with EcoRI and SalI at both ends: Ehm2 Tag Forward: TAGAATTCATGCTGCGGTTCTGC and Ehm2 Tag Reverse: GAGCGTCGACTCAAAGTG-CAGTCAT from LNCaP cell line. The Ehm2 PCR fragment was cloned into EcoRI/SalI site of pCMV-Tag2B vector (Stratagene, La Jolla, CA), which has T3 and T7 at both ends of the insert to be used to generate sense and anti-sense RNA probes. Digoxigenin-labeled antisense and sense RNA probes were synthesized using MAXIsript in vitro RNA transcription kit (Ambion, Inc., Austin, TX) and a linearized plasmid with the Ehm2 gene fragment as templates. Tissue slides containing 12 prostate tissues were used. In situ hybridization was carried out by standard procedures. Briefly, prostate tissues were de-waxed in xylene, 3  $\times$  10 min, hydrated, digested with 40  $\mu$ g/ml proteinase K for 7 min at room temperature, fixed in 4% paraformaldehyde for 20 min. One microgram Dig-labeled probe was used for 1 ml hybridization buffer (50% formamide, 10% SSC). Hybridization was performed at 70°C overnight, after which slides were sequentially washed by Dig Wash and Block Buffer Set (Roche Diagnostics, Indianapolis, IN) following manufacturer's protocol. Anti-digoxigenin antibody (1:2,000) was used to detect the signal and NBT/BCIP was used as substrate for color development (Boehringer Mannheim, Germany).

### Immunohistochemistry Using Tissue Microarray

A prostate cancer tissue array was obtained from the Baylor Prostate Cancer SPORC containing 32 cases prostate cancer from men undergoing radical prostatectomy. Each case was represented by five 0.2-mm punches of normal peripheral zone or cancer tissue. Immunohistochemistry was carried out as described previously [22]. Primary antibody incubation was carried out at 4°C overnight at a 1:300 dilution using a goat polyclonal anti-Ehm2 antibody (F21; Santa Cruz Biotechnology), which was raised against a peptide mapping near the carboxy terminus of Ehm2 of mouse origin followed by the avidin-biotin peroxidase complex procedure (Vector Laboratories, Inc.,

Burlingame, CA). Slides were digitized and staining was evaluated semi-quantitatively in normal epithelium and prostate cancer by a pathologist (M.I.). Expression in normal or neoplastic prostatic epithelium was scored according to the intensity and percentage of stained tumor and normal prostate glands as described previously. Atrophic or metaplastic glands were not scored. Weak staining was graded as 1, intermediate staining was considered as 2, and strong staining was considered as 3. As for the percentage of stained glands, 1 stands for 1 ~ 33% glands with staining, 2 for 34 ~ 66%, and 3 for 67 ~ 100%, respectively. The staining index was calculated as the product of the intensity and percent score, resulting in a staining index ranging from 0 (no staining) to 9 (extensive, strong staining). The mean score of the five cores was then calculated for each case. Of the 32 cases, 27 normal and 30 cancer cases gave usable data, with some cases lost due to technical artifacts or absence of appropriate epithelium after multiple previous sections. In two cases, no clinical follow-up was available. As a negative control, a fivefold molar excess of blocking peptide incubated for 2 hr at room temperature with antibody to generate a negative control antibody. Immunohistochemistry with this pre-incubated antibody showed no staining (data not shown).

### Cloning of Ehm2 cDNA and Transient Transfection

The 1,557-bp Ehm2 cDNA was generated by PCR using total RNA isolated from dihydrotestosterone-treated HT-AR1 cells [16] and a 5' primer with the sequence 5'-ATGCTGCGGTTCTGCGCC GGACC-3' that also included a single FLAG epitope coding sequence and a HindIII cloning site, and a 3' primer containing the EHM2 sequence 5'-AGTCTCTCAAAGT GCAGTCAT-3' and a BamHI cloning site. The PCR reaction was performed using the GC-RICH PCR system from Roche Diagnostics following the manufacturer's recommendations. The PCR product was digested with HindIII and BamHI and cloned into the pCDNA 4/TO vector (Invitrogen) and sequenced. For expression studies, the full length Ehm2 fragment was excised with HindIII and BamHI and cloned into the pCEP4 expression vector. A shorter Ehm2 fragment was also cloned from LNCaP cells which lacked 258 bp of 5' sequence compared to full length Ehm2 cDNA. Transient transfection of 293T cells using both Ehm2 expression vectors verified the expression of full length and short form Ehm2 proteins by Western blot using an anti-Flag M2 antibody prior to transfection of PNT1a cells. PNT1a cells were plated at  $2 \times 10^6$  cells per 100-mm dish 24 hr before transfection and transfected with 20  $\mu$ l Fugene6 (Invitrogen) and 7  $\mu$ g of plasmid (pCEP4-

Ehm2, pCMV-Tag2B-Ehm2 or control vector) for 5 hr in a total volume of 4 ml of RPMI 1640 medium without serum. One milliliter of FBS was then added to each dish to achieve a final serum concentration of 20%. Cells were refed with complete medium on Day 2. After an additional 24 hr incubation, cells were used to perform collagen IV assays as described below or used for RNA extraction.

### Generation of Ehm2 SiRNA and Transfection

Silencer<sup>TM</sup> siRNA Cocktail Kit (Ambion) was used to decrease the expression of Ehm2 in LAPC4 cell line. To prepare the DNA template for generating SiEhm2, amplification strategy of a single PCR with the T7 promoter appended to both PCR primers was used. Primers, which amplified a fragment of ~400 bp located at 5' end of Ehm2 sequence, were used. siEhm2F: 5'-TAATACGACTCACTATAGGGAGG AT GCTGCGGTTCTG-3'; siEhm2R: 5'-TAATACGACTCACTATAGGGCAAGCT CCGCTTGTAAGAC-3'. To generate SiRNA, the RNA transcript from this PCR product was digested following the manufacturer's instruction protocol to yield 12–15 bp oligonucleotides, which were stored at a concentration of 20  $\mu$ M at  $-20^{\circ}\text{C}$ . LAPC4 cells were treated with this Si-Ehm2 oligonucleotides mix at 200 nM using Oligofectamine<sup>TM</sup> Reagent (Invitrogen). The LAPC4 cells were seeded at  $3 \times 10^5$  in 6-well plates 24 hr before the transfection in Opti-MEM I Reduced Serum Medium. Four hours after initiation of transfection, growth medium containing three times the normal concentration of serum was added without removing the transfection mixture. RNA was extracted 24 hr post-transfection and Ehm2 transcript level determined by RT-PCR or cells plated for to determine collagen IV adhesion as described below.

### SiFGFR-4 in LNCaP Cells

To investigate if Ehm2 expression can be modulated by reduced FGFR-4 expression, we generated a LNCaP cell line with stably reduced FGFR-4 expression using Block-iT<sup>TM</sup> Lentiviral RNAi Expression System (Invitrogen). Primers used for Lentiviral construct were: SiR4Top 5'-CA CCGCATA GGGACCTCTCGA ATATT CGAAAATATTTCGAGAGGTCCCTATGC-3' SiR4Bot 5'-AAAAGCATAGGGACCTCTCGAATATT TTCGA ATATTTCGAGAGGTCCCTATGC-3'. Lentiviral construct was generated according to manufacturer's instruction. Lentivirus which stably expresses the shRNA of FGFR-4 was produced by 293FT cells. LNCaP cells were infected with lentivirus and selected in Blasticidin (2  $\mu$ g/ml) for 2 weeks. RNAs and protein lysates were prepared from LNCaP and LNCaP

Si-FGFR-4 cells, and were used for real-time PCR and Western Blot.

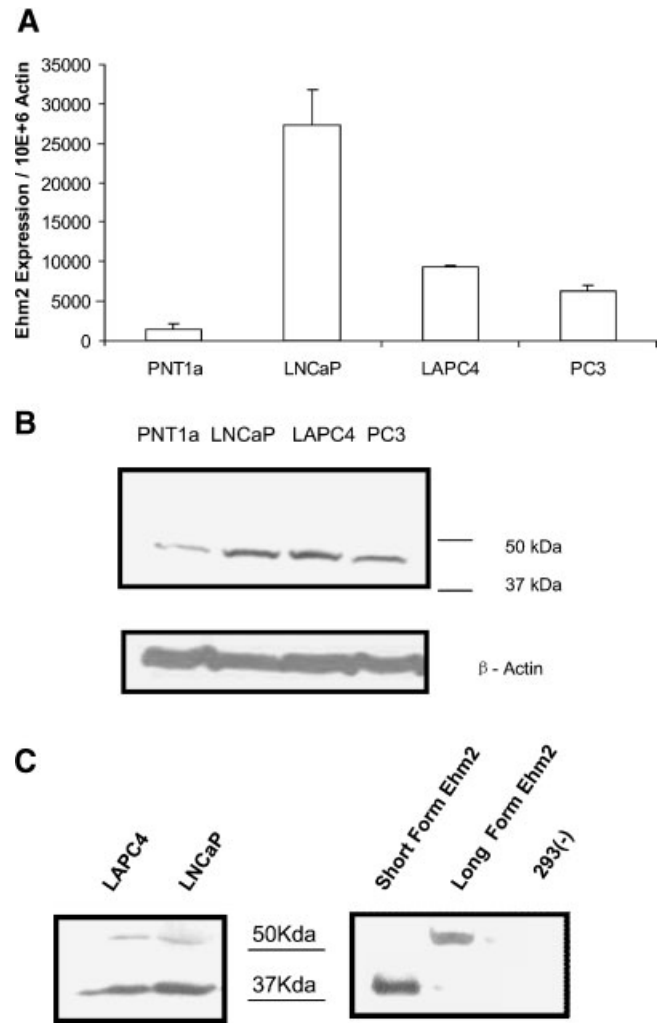
### Cell Adhesion Assay

For cell-substrate adhesion assay, 6-well plates coated with collagen IV were purchased from BD Biosciences. Transfected ( $5 \times 10^5$ ) PNT1a or LAPC4 cells were seeded to each well, after incubating at 37°C for 1 hr, non-adherent cells were removed by washing with PBS. Adherent cells were incubated for another 2 hr before counting. Experiments were done in triplicate and repeated three times.

## RESULTS

### Expression Level of Ehm2 in Immortalized Prostate Epithelial Cells and Prostate Cancer Cell Lines

To determine whether Ehm2 expression is increased in prostate cancer, we initially carried out the quantitative real-time PCR analysis using reverse transcribed RNAs from an immortalized cell line derived from benign prostatic epithelial cells (PNT1a) and three prostate cancer cell lines (PC-3, LNCaP, and LAPC4). Compared to PNT1a, higher levels of Ehm2 mRNA expression could be detected in all three prostate cancer cell lines, with the strongest expression in the androgen dependent, androgen receptor expressing LNCaP and LAPC4 cells (Fig. 1A). To confirm these findings at the protein level, we performed Western blots on protein extracts of these same four cell lines using an anti-Ehm2 antibody. A single band of approximately 43 kDa was seen in all four cell lines. This band was not seen in blots using antibody pre-incubated with the immunizing peptide (data not shown). The band is smaller than the predicted size of the full-length Ehm2 protein (57 kDa), implying the possibility of alternative translation initiation or other post-transcriptional events resulting in a smaller Ehm2 protein in prostate epithelial cells. Longer exposures of Western blots of LAPC4 and LNCaP cells revealed a weak band of approximately 57 kDa, consistent with the full length form of Ehm2 (Fig. 1C). The longer and shorter forms of Ehm2 seen in the prostate cancer cell lines on Western blots with anti-Ehm2 antibody comigrated with full length and truncated Ehm2 transiently transfected into 293T cells and detected by anti-Flag antibody directed against a Flag-epitope engineered into these constructs. It should be noted that the 5' portion of the Ehm2 mRNA is very GC-rich. Such GC-rich regions can be associated with stable secondary structures which in the appropriate cellular context, promote internal ribosome entry [23] that in this case could result in a truncated protein.

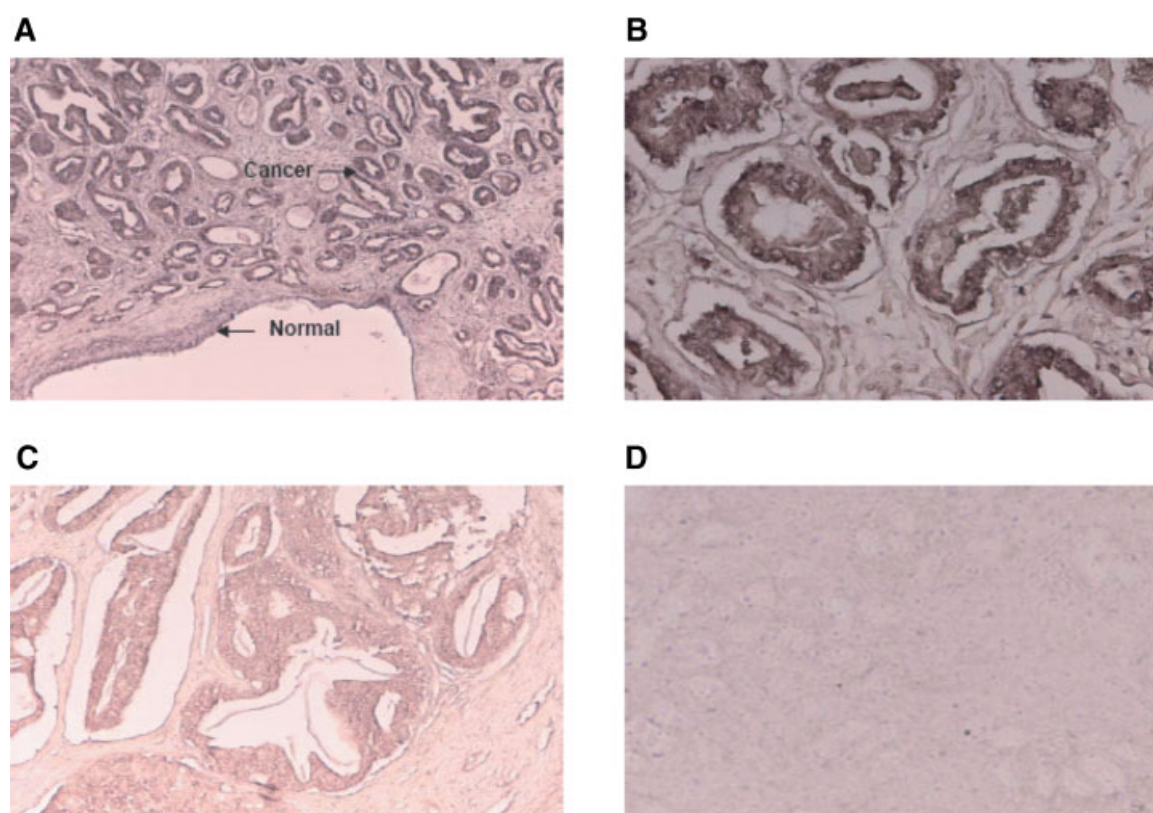


**Fig. 1.** Ehm2 expression in prostate cell lines. **A:** Expression of Ehm2 mRNA was determined by quantitative RT-PCR and normalized to  $\beta$ -actin levels and expressed as mean  $\pm$  SD for duplicate experiments. PNT1a is an immortalized cell line derived from normal prostatic epithelium. LNCaP, LAPC4, and PC3 are prostate cancer cell lines. **B:** Western blot with anti-Ehm2 antibody or control anti- $\beta$ -actin antibody. **C:** Longer exposure of Western blot of LAPC4 and LNCaP extracts demonstrating expression of full-length and truncated Ehm2 protein. Western blot of extracts of 293T cells transiently transfected with short or long Ehm2 cDNAs with anti-Flag antibody is shown on the left.

### Localization of Expression of Ehm2 mRNA by In Situ Hybridization

To define the localization of Ehm2 mRNA in normal and cancer prostate tissues, in situ hybridization was carried out in 12 human prostate tissue samples. As shown in Figure 2, Ehm2 is expressed in normal and malignant prostatic epithelium with normal prostate epithelium showing a lower level of Ehm2 mRNA compared to the cancer epithelial cells in the same tissue (Fig. 2A, arrows). Ehm2 expression was





**Fig. 2.** Localization of Ehm2 expression in human prostate tissues using in situ hybridization. Ehm2 mRNA expression in human prostate tissues was determined by in situ hybridization as described in Materials and Methods. **A:** Prostate cancer and adjacent normal epithelium (Original magnification, 100 $\times$ ). **B:** Strong expression of Ehm2 in prostate cancer tissues (400 $\times$ ). **C:** Moderate Ehm2 expression in prostate cancer (100 $\times$ ). **D:** In situ hybridization using a sense probe of Ehm2 as a negative control (100 $\times$ ).

predominantly in the epithelial cells of the prostate gland, with little expression in the stromal tissues (Fig. 2A). DIG-labeled sense probe of Ehm2 was used as a negative control for in situ hybridization and very low level of signals were detected (Fig. 2D), confirming the specificity of the in situ hybridization.

#### Determination of Ehm2 RNA Levels by Quantitative Real-Time PCR in Normal Prostate and Prostate Cancer Tissues

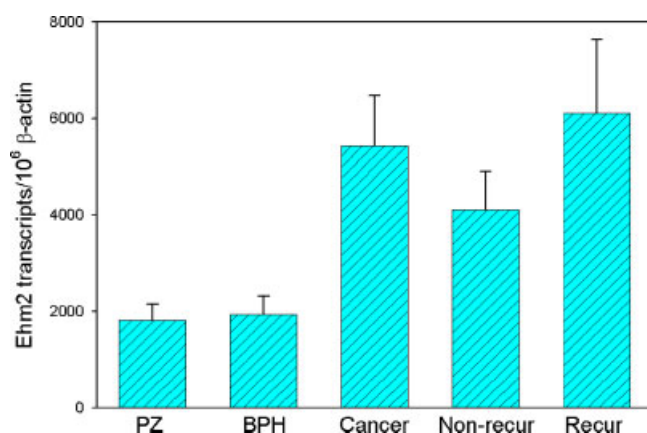
Our data on Ehm2 expression in cell lines indicated that Ehm2 is expressed in prostate epithelial cells and is present at higher levels in prostate cancer cells. To confirm this observation in vivo using a quantitative technique, we examined the Ehm2 transcript expression in 19 normal peripheral zone tissues (PZ), 8 hyperplastic transition zone samples (BPH), and 59 cancer tissues (containing at least 70% cancer) using quantitative RT-PCR. As seen in Figure 3, Ehm2 mRNA levels were about threefold higher in the cancer tissues ( $5,430 \pm 1,044/10^6$   $\beta$ -Actin; mean  $\pm$  SEM) when compared to normal peripheral zone tissues ( $1,824 \pm 337/$

$10^6$   $\beta$ -Actin). This difference was highly statically significant ( $P < 0.001$ , Mann–Whitney Rank Sum Test). BPH tissues showed low expression level of Ehm2 ( $1,942 \pm 385/10^6$   $\beta$ -Actin), similar to normal peripheral zone tissues. Ehm2 expression was higher in cancers from men that subsequently developed PSA recurrence, although this difference was not statistically significant.

#### Immunohistochemical Analysis of Ehm2 Expression Using Tissue Microarrays

To analyze expression of Ehm2 at the protein level in vivo, we performed immunohistochemical analysis of Ehm2 expression using a prostate cancer tissue microarray containing 32 matched normal and prostate cancer tissues (Fig. 4). Staining was almost exclusively seen in benign and malignant epithelial cells and was variable between different cases. Stained slides were digitized and scored both for extent of staining (scale of 0–3) and intensity of staining (scale of 0–3; see Materials and Methods). An average staining index was calculated from the extent of staining score for the



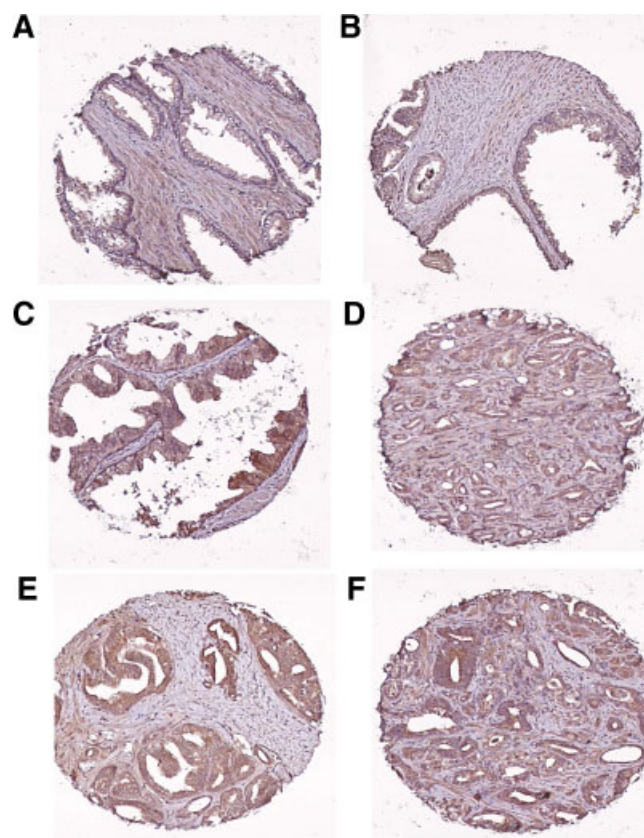


**Fig. 3.** Ehm2 mRNA expression in benign prostate and prostate cancer tissues as determined by quantitative real-time PCR. Expression of Ehm2 mRNA was determined by quantitative RT-PCR and normalized to  $\beta$ -actin levels. PZ, normal peripheral zone; BPH, hyperplastic transition zone; Cancer, all prostate cancers; Non-recur, prostate cancers with no PSA recurrence within 5 years of follow-up; Recur, prostate cancers with PSA recurrence within 5 years after radical prostatectomy. The Ehm2 expression levels are displayed as the copy number of Ehm2 transcripts per 10<sup>6</sup>  $\beta$ -actin transcripts (mean  $\pm$  standard error of the mean). The data are a representative of one of two separate experiments. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

three cores multiplied by the staining intensity score so that the staining index ranged from 0 (no staining) to 9 (extensive, strong staining). Normal prostate epithelium showed a staining index of  $6.3 \pm 0.38$  (mean  $\pm$  SEM) while the cancer tissues had a staining index  $7.4 \pm 0.29$ . This difference was statistically significant ( $P < 0.05$ , Mann–Whitney). Overall, 27% of normal tissues showed low staining (index  $< 6.0$ ) while only 10% of cancers had low staining. In contrast, 40% of cancers showed maximal staining intensity (index = 9) versus 8% of normal epithelium. Of note was the finding that none of the three cancers with low Ehm2 staining (staining index  $< 6.0$ ) had a PSA recurrence, while 19 of 25 cases with recurrence had moderate to strong Ehm2 staining (staining index 6–9). This difference is statistically significant ( $P = 0.03$ , Fisher exact test).

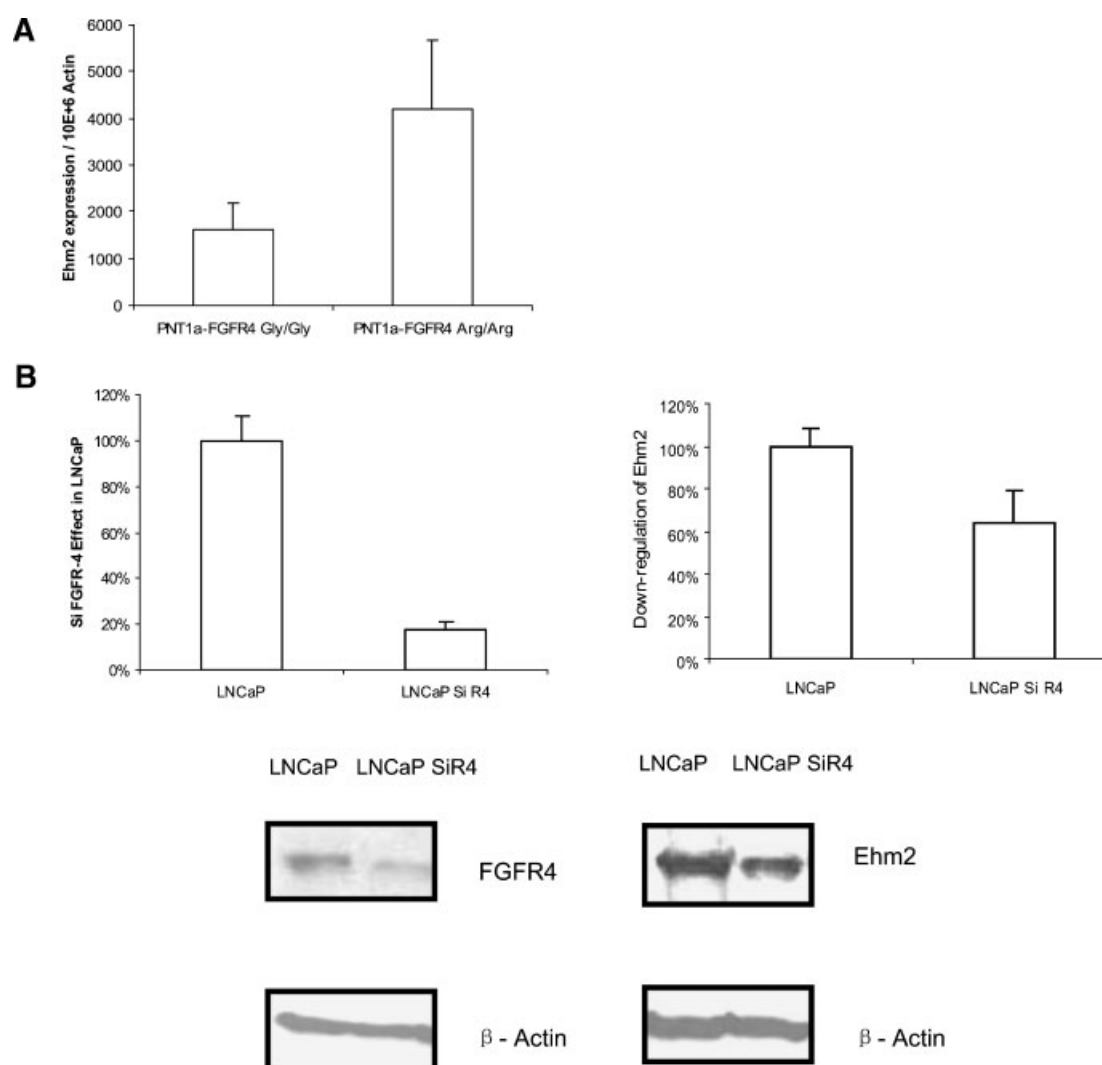
#### The FGF Receptor-4 Arg<sup>388</sup> Variant Increases Ehm2 Expression

We have shown previously that prostate cancers arising in men bearing the FGFR-4 Arg<sup>388</sup> polymorphic have increased rates of pelvic lymph node metastasis following radical prostatectomy [7]. To determine if Ehm2 may play a role in this increased rate of metastasis, we measured expression of Ehm2 transcripts in pooled, stably transfected PNT1a cells lines



**Fig. 4.** Immunohistochemical determination of Ehm2 expression in prostate tissues. Tissue sections from radical prostatectomies were analyzed by immunohistochemistry with an anti-Ehm2 antibody as described in Materials and Methods using a tissue microarray. **A:** Normal prostate with weak Ehm2 staining of epithelium. Some staining of smooth muscle is also present. **B:** Normal prostate with moderate Ehm2 staining. **C:** High grade prostatic intraepithelial neoplasia with strong Ehm2 staining. **D:** Prostate cancer with moderate Ehm2 staining. **E, F:** Prostate cancers with strong Ehm2 staining.

expressing either FGFR-4 Arg<sup>388</sup> or the more common Gly<sup>388</sup> variant. As described previously, these cell lines express similar levels of FGFR-4 [7]. Analysis of Ehm2 expression in these two cell lines by quantitative RT-PCR reveals that cells expressing the FGFR-4 Arg<sup>388</sup> variant had 2.6-fold higher levels of Ehm2 transcripts than cells expressing the FGFR-4 Gly<sup>388</sup> variant (Fig. 5A). The PNT1a cells expressing the FGFR-4 Gly<sup>388</sup> variant had Ehm2 transcript levels similar to parental PNT1a cells (Fig. 1A). To confirm that Ehm2 protein levels are regulated by FGFR-4 protein, we used a SiRNA directed against FGFR-4 to downregulate FGFR-4 expression in LNCaP cells which have an Arg<sup>388</sup>/Gly<sup>388</sup> heterozygous genotype. Western blots of protein extracts after transfection with the FGFR-4 SiRNA show marked decreases in both FGFR-4 and Ehm2 RNA and protein expression (Fig. 5B).



**Fig. 5.** Regulation of Ehm2 expression by FGFR-4 Arg<sup>388</sup>. **A:** Expression of Ehm2 mRNA was determined by quantitative RT-PCR and normalized to  $\beta$ -actin levels in RNAs from PNT1a cells overexpressing either the FGFR-4 Arg<sup>388</sup> or Gly<sup>388</sup> variant. Values are mean  $\pm$  SD for duplicate experiments. **B:** Expression of FGFR-4 and Ehm2 mRNA and protein as determined by quantitative RT-PCR (mean  $\pm$  SD) and Western blots in cells stably transduced with a FGFR-4 SiRNA.

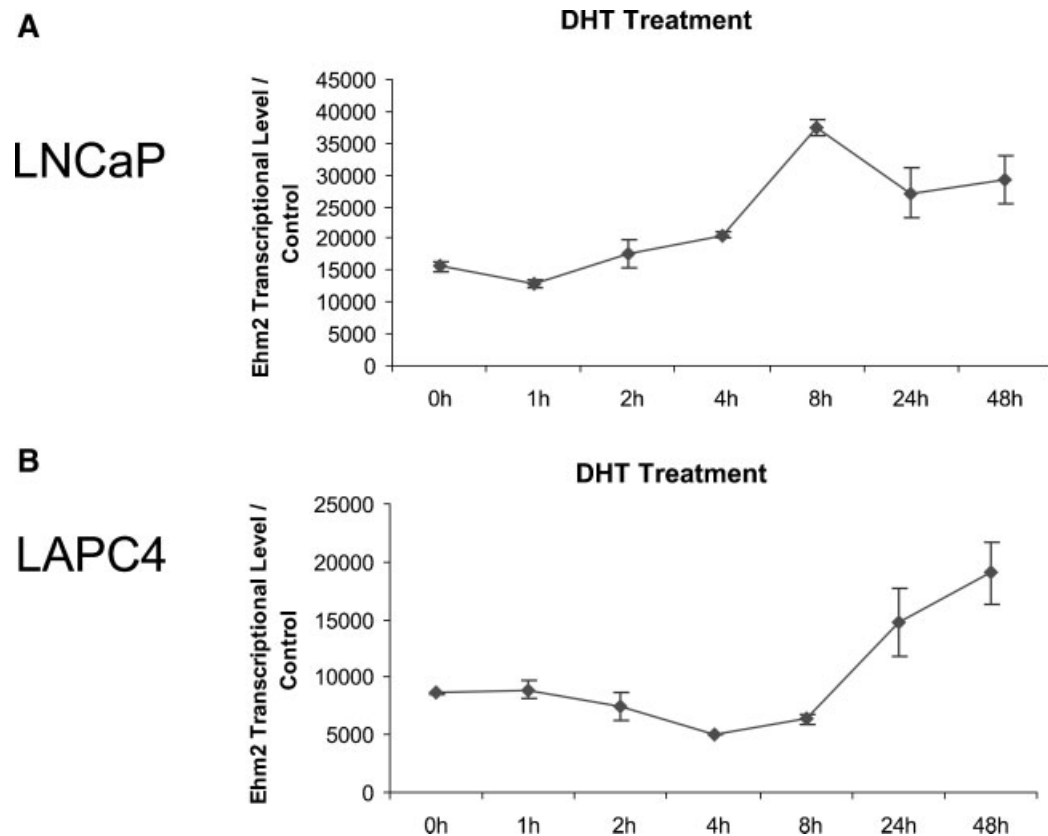
Thus, FGFR-4 Arg<sup>388</sup> expression results in increased expression of Ehm2.

#### Expression of Ehm2 Is Increased by Androgens

Based on the observation that androgens can increase expression of Ehm2 in other cell lines [16,24], and the observed higher expression of Ehm2 in androgen receptor expressing prostate cancer cell lines (Fig. 1), we tested whether there was a similar modulation of Ehm2 expression in prostate cancer cells. As shown in Figure 6, androgen treatment resulted in a twofold increase in Ehm2 transcript levels in both LNCaP and LAPC4 cells within 8–24 hr after treatment of these androgen receptor expressing cells with androgen.

#### Ehm2 Expression Modulates Cell Adhesion to Collagen IV

Ehm2 may regulate cytoskeleton interactions with transmembrane proteins and thus affect cancer cell adhesion [15]. In particular, alterations of FGFR-4 have been linked to decreased collagen IV adhesion in other tumors [24]. To evaluate the impact of Ehm2 on collagen IV adhesion, we carried out two series of complementary experiments. PNT1a cells, which express low levels of Ehm2, were transiently transfected with an expression vector containing the full length Ehm2 cDNA. Expression of Ehm2 was increased eightfold in the transfected cells and this was associated with a  $24 \pm 2.7\%$  (mean  $\pm$  SEM,  $n = 3$ ) decrease in adhesion to collagen IV (Fig. 7A). Since the smaller



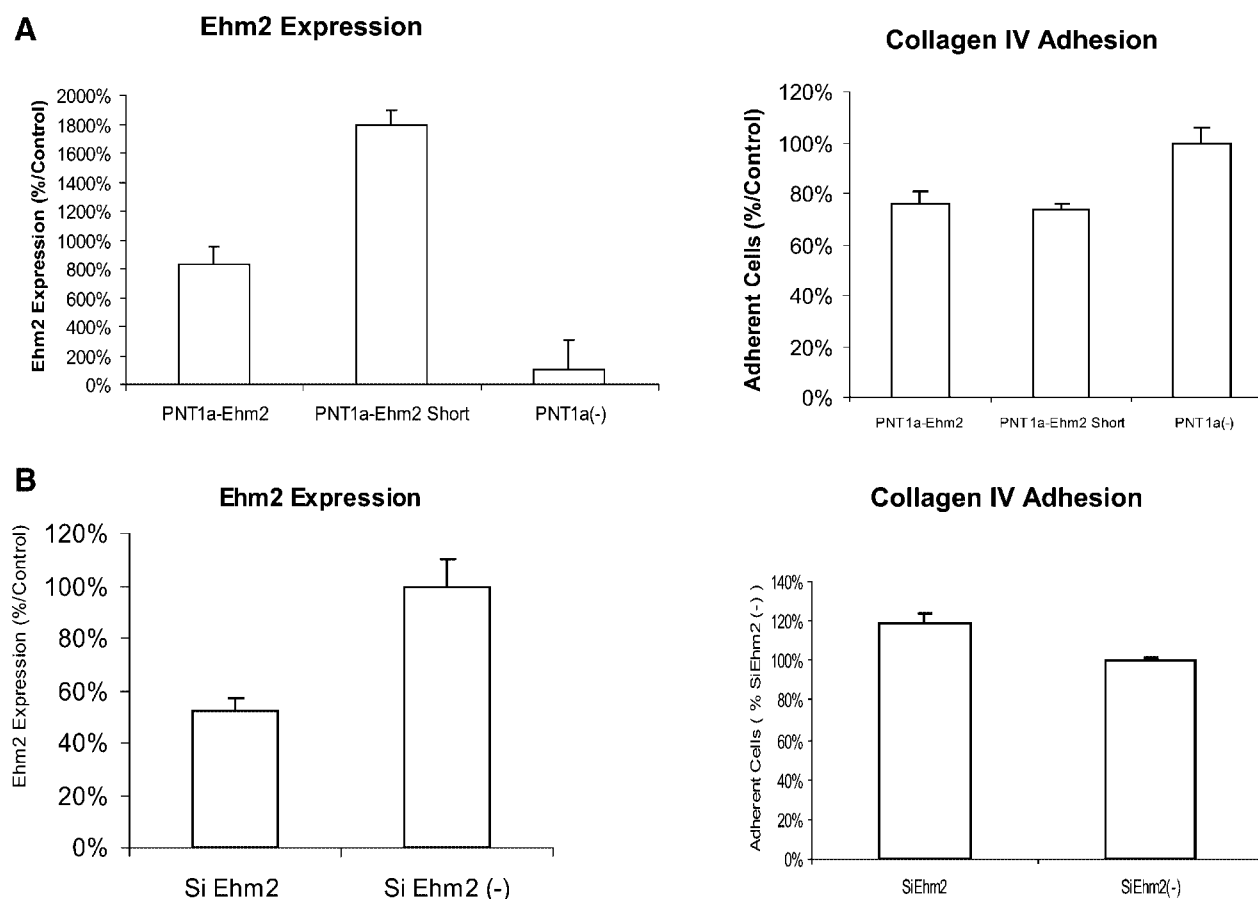
**Fig. 6.** Ehm2 expression can be induced by androgens. LNCaP(A) and LAPC4(B) cells were grown in media with charcoal stripped serum for 24 hr, then cells were refed with media containing 10 nM DHT. Cells were collected at the indicated times, with media changed at 24 hr. Ehm2 RNA levels were determined by quantitative RT-PCR and normalized to  $\beta$ -actin expression. Values are mean  $\pm$  SD for duplicate experiments.

form of the Ehm2 protein is the predominant form expressed in prostate cancer, we transiently expressed a truncated cDNA expressing only the smaller Ehm2 protein in PNT1a cells. Expression of this shorter form resulted in similar decrease in collagen IV adhesion ( $26.3 \pm 1.3\%$ , mean  $\pm$  SEM,  $n = 3$ ). We then decreased Ehm2 mRNA in the LAPC4 cell line, which showed the second strongest Ehm2 expression among tested prostate cell lines, using a mixed SiRNA directed against the 5' portion of Ehm2. As shown in Figure 7B, 24 hr after SiRNA transfection, Ehm2 expression showed an approximately 50% decrease in Ehm2 transcript level, while adhesion to collagen IV increased  $19 \pm 0.9\%$  (mean  $\pm$  SEM,  $n = 3$ ) compared to control cells. These results indicate that increased Ehm2 expression is associated with decreased adhesion of prostatic epithelial cells to collagen IV.

## DISCUSSION

The *Ehm2* gene was originally identified by differential display analysis as a gene that was upregulated in more highly metastatic clones of K-1735 and B16 murine melanoma cells [14,15]. Structural analysis of

Ehm2 revealed a FERM domain (F for 4.1 protein, E for ezrin, R for radixin, and M for moesin), which is highly conserved among different species and has been showed to be involved in the linkage of cytoplasmic proteins to the membrane [26]. For example, the cytoskeletal protein talin contains a FERM domain which plays an important role in integrin activation [27–29]. The related ERM proteins play a role in the interaction of transmembrane proteins such as ICAMs and CD44 with the actin cytoskeleton [26]. Other FERM domain proteins are cell signaling proteins such as tyrosine phosphatases [30] and focal adhesion kinase [31]. FERM domain proteins, such as NF2, can act as tumor suppressor genes [32] while others, such as URP1, are upregulated in cancer [33] and may play a role in tumor progression. Thus, FERM domain proteins can mediate a variety of critical processes at the cell membrane and may play a role as both repressors and promoters of tumor initiation and progression. Interestingly, the *Drosopholia* ortholog of Ehm2, called Yurt [34], has been shown to be required for epithelial cell migration during embryogenesis, suggesting that its mammalian homolog may play a role in cancer metastasis by altering cell migratory



**Fig. 7.** Ehm2 and collagen IV adhesion. **A:** Effects of overexpression of Ehm2 in PNT1a cells on collagen IV adhesion. PNT1a cells were transiently transfected with Ehm2 cDNA or empty vector (pCEP4). Ehm2 expression was determined by quantitative RT-PCR of RNAs extracted 24 hr after transfection. Range of triplicate determinations is shown. Transfected cells were plated on collagen IV coated plates and adhesion determined as described in Materials and Methods. Adhesion is expressed as percentage of control. **B:** Effect of downregulation of Ehm2 expression in LAPC4 cells on collagen IV adhesion. LAPC4 cells were transiently transfected with Ehm2 SiRNA mixture or mock transfected. Ehm2 expression was determined by quantitative RT-PCR of RNAs extracted 24 hr after transfection. Range of triplicate determinations is shown. Transfected cells were plated on collagen IV-coated plates and adhesion determined as described in Materials and Methods. Adhesion is expressed as percentage of control.

properties. However, the biological function of Ehm2 in human prostate cells is unknown.

We have found that Ehm2 is expressed in normal prostate, prostate cancer, and cell lines derived from either normal or malignant prostate epithelial cells. Chauhan et al. [16] have previously reported expression of Ehm2 in normal prostate by Northern blotting, consistent with our observations. Based on in situ hybridization and immunohistochemistry, Ehm2 is expressed almost exclusively in luminal epithelial cells and cancer cells in the prostate, which is consistent with it being an androgen-regulated target gene [16,25]. We found by quantitative RT-PCR and immunohistochemistry, that Ehm2 is expressed at higher levels in prostate cancer cells. Low Ehm2 expression by immunohistochemistry was associated with decreased risk of biochemical recurrence. Such biochemical recurrence is associated with development of metastasis and death

from prostate cancer in a significant fraction of cases, while almost all men without evidence of PSA recurrence within 5 years of radical prostatectomy will remain disease-free [35]. Thus, Ehm2 is expressed at higher levels in prostate cancer and higher expression is associated with more aggressive disease.

We have shown previously that prostate cancer arising in men with a germline polymorphism of FGFR-4, which results in substitution of arginine for glycine at amino acid 388, is significantly more aggressive in that these men have higher rates of pelvic lymph node metastasis and biochemical recurrence and increased invasiveness in vitro. We report here that the FGFR-4 Arg<sup>388</sup> polymorphism is specifically associated with increased Ehm2 expression. At least part of the increased aggressiveness of prostate cancers expressing the FGFR-4 Arg<sup>388</sup> variant may be due to increased Ehm2 expression in these cancers. Our

studies indicate that Ehm2 expression can decrease adhesion to collagen IV. The degree of loss of adhesiveness is correlated with metastatic ability in some cancers. For example, in breast cancer decreased adhesion of primary breast cancer cells to collagen IV and other matrix proteins was significantly associated with lymph node metastasis [36]. The decrease in collagen IV adhesion in cells expressing Ehm2 is thus consistent with the more aggressive clinical behavior of prostate cancer cells expressing higher levels of Ehm2.

Ezzat et al. [24] have reported that a cytoplasmic FGFR-4 variant arising from a cryptic promoter (ptd-FGFR-4) is expressed in pituitary adenomas and that expression of this variant resulted in increased invasiveness of pituitary cells. Expression of the ptd-FGFR-4 variant decreased adhesion to collagen IV in pituitary cells and NIH3T3 fibroblasts compared to full length FGFR-4 in a ligand independent manner. These authors provide evidence that expression of the variant FGFR-4 leads to disruption of formation of NCAM and N-cadherin complexes in pituitary cells which could affect adhesion to collagen IV. On the other hand, Coppolino and Dedhar [37] have shown that inhibition of mitogen-activated protein kinase enhances PC3 cell attachment to collagen IV, implying that altered signal transduction from growth factor receptors can modulate collagen IV adhesion. Clearly, further mechanistic studies are needed of the role of FGFR-4 in cell adhesion and the possible participation of Ehm2 in this process. However, the finding of both increased invasiveness and decreased collagen IV adhesion in pituitary cells expressing the ptd-FGFR-4 variant has clear parallels to our findings regarding the FGFR-4 Arg<sup>388</sup> variant and Ehm2 in prostate cancer.

Based on our studies, expression of Ehm2 is controlled by multiple factors. On average, both in vitro and in vivo, transformed prostatic epithelial cells express two- to threefold higher levels of Ehm2 than untransformed cells. In addition, androgen can induce Ehm2 approximately twofold in androgen receptor expressing prostate cancer cells. Finally, the presence of the FGFR-4 Arg<sup>388</sup> variant is also associated with two- to threefold increase in Ehm2 expression. The mechanism(s) by which FGR-4 Arg<sup>388</sup> and androgen can induce Ehm2 mRNA expression is unclear. There are indications that these mechanisms may be additive. Comparing the expression of Ehm2 in the androgen receptor expressing cell lines, the LNCaP cell line has an Arg<sup>388</sup>/Gly<sup>388</sup> genotype and has a 2.5-fold higher expression of Ehm2 compared to LAPC4, which has a Gly<sup>388</sup>/Gly<sup>388</sup> genotype. Similarly, for the AR negative cell lines, PC3, which has an Arg<sup>388</sup>/Arg<sup>388</sup> genotype, expresses 2.1-fold more Ehm2 than DU145, which has a Gly<sup>388</sup>/Gly<sup>388</sup> genotype. Detailed studies of the underlying mechanisms, either direct or indirect, that

control expression Ehm2 in response to androgens and FGFR-4 expression are clearly needed.

In summary, Ehm2 is increased in prostate cancer and higher expression is associated with increased risk of biochemical recurrence following radical prostatectomy. Concordant with this finding, Ehm2 expression is increased in prostate cells expressing the FGFR-4 Arg<sup>388</sup> variant, which is also associated with biochemical recurrence. At the cellular level, increased Ehm2 expression results in decreased adhesion of cells to collagen IV. Further mechanistic studies defining the molecular mechanisms controlling Ehm2 expression and its role in cell adhesion are needed to clarify the role of this protein in the progression of prostate cancer.

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